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Examination of food chain-derived *Listeria monocytogenes* of different serotypes reveals considerable diversity in *inlA* genotypes, mutability, and adaptation to cold temperature

Running title: *inlA*, mutability, and cold growth of *L. monocytogenes*

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ABSTRACT

Listeria monocytogenes belonging to serotypes 1/2a and 4b are frequently linked to listeriosis. Whilst *inlA* mutations leading to premature stop codons (PMSC) and attenuated virulence are common in 1/2a, they are rare in serotype 4b. We observed PMSCs in 35% of *L. monocytogenes* isolates (n=54) recovered from the British Columbia food supply, including serotypes 1/2a (30%), 1/2c (100%), and 3a (100%), and a 3-codon deletion (amino acid positions 738-740) seen in 57% of 4b isolates from fish processing facilities. Caco-2 invasion assays showed two isolates with the deletion were significantly more invasive than EGD-SmR (p<0.0001), and were either similar (FF19-1) or more invasive (FE13-1) than a clinical control strain (08-5578) (p=0.006). To examine whether serotype 1/2a was more likely to acquire mutations than other serotypes, strains were plated on agar with rifampicin, revealing 4b isolates to be significantly more mutable than 1/2a, 1/2c, and 3a serotypes (p=0.0002). We also examined the ability of 33 strains to adapt to cold temperature following downshift from 37 to 4°C. Overall, three distinct cold-adapting groups (CAG) were observed: 46% were fast (<70 h), 39% intermediate (70-200 h), and 15% slow adaptors (>200 h). Intermediate CAG strains (70%) more frequently possessed *inlA* PMSCs compared to fast (20%) and slow (10%) CAGs; in contrast, 87% of fast-adaptors lacked *inlA* PMSCs. In conclusion, we report food chain-derived 1/2a and 4b serotypes with a 3-codon deletion possessing invasive behavior, and the novel association of *inlA* genotypes encoding a full-length InlA with fast cold-adaptation phenotypes.

Listeria monocytogenes is an environmentally ubiquitous organism that frequently contaminates food processing environments. It is estimated that 99% of listeriosis cases are transmitted through the consumption of contaminated food (32, 42, 44). In healthy individuals, *L. monocytogenes* infections are rare, restricted to the gastrointestinal environment, self-limiting, and manifest as gastroenteritis and/or mild flu-like symptoms. In contrast, in susceptible populations (e.g., neonates, the elderly, and immunocompromised), infections become invasive, leading to encephalitis, meningitis, septicemia, and/or spontaneous abortions during the last trimester of pregnancy (31); mortality rates for invasive listeriosis typically range between 20 to 40% (12, 22, 49).

Although there are 13 *L. monocytogenes* serotypes, 1/2a, 1/2b, and 4b account for the majority of human disease (9, 44). Historically, lineage I 4b strains have been over-represented in clinical listeriosis cases and are less frequently recovered from foods (9, 20, 24). However, over the past decade, lineage II 1/2a strains typically over-represented in food and environmental samples (7, 20, 44) have been frequently linked to human disease, causing notable outbreaks in Switzerland (4), the United Kingdom, and two 2008 outbreaks in Canada (17, 49). With regards to the latter, 1/2a strains comprise the majority of Canadian clinical isolates, followed by 4b (10). Reasons for the prevalence of 1/2a strains in human disease in Canada may be linked to a recently identified clonal complex/epidemic 1/2a clone that was identified as a recurring cause of sporadic listeriosis since 1988 (26). Within this complex, the majority of 1/2a strains were found to possess the LGI1 genomic island which was first identified in an outbreak strain linked to 23 deaths (18), and more recently recovered from smoked salmon in British Columbia (BC) (28).

77 Over the past decade, sequence analysis of *inlA*, which encodes a membrane-
78 bound protein facilitating invasion into non-professional phagocytes, reveals a significant
79 proportion (45%) of strains recovered from ready-to-eat (RTE) food possess mutations
80 resulting in a premature stop codon (PMSC) (34, 47, 48). Strains with *inlA* PMSCs
81 produce either a truncated or non-secreted InlA, resulting in virulence attenuated
82 phenotypes as measured by *in vitro* cell assays (13, 21, 25, 33, 41) and mammalian
83 models (34, 40, 46).

84 Whilst infrequent reporting of PMSCs in 4b strains may reflect their over-
85 representation in clinical listeriosis, the contrary is postulated for 1/2a strains. Being
86 frequently recovered in food and food production environments, positive selection for
87 strains with PMSCs may serve as a phase switch that is important for environmental
88 survival (35). In-line with this, it has been suggested that lineage II 1/2a strains are better
89 able to survive conditions associated with the food chain. Notably, 1/2a and 1/2c (lineage
90 II) more frequently possess PMSCs than 1/2b or 3b serotypes, with 4b strains rarely
91 having PMSCs (35). In general, serotype 4b appears more recalcitrant to genetic flux,
92 being less likely to acquire or possess plasmids and to experience homologous
93 recombination events that may afford rapid adaptation to niche-specific stresses.

94 In this study, we were interested in observing how *L. monocytogenes* with
95 differing *inlA* genotypes respond to food chain-relevant conditions. In particular, the
96 reliance on refrigeration to maintain the quality of fresh and RTE foods makes cold
97 temperature a suitable and relevant parameter to examine. To this end, we ascertained the
98 nature and prevalence of *inlA* genotypes in *L. monocytogenes* serotypes recovered from
99 food production environments and food in BC, Canada, and assessed the capacity of

100 strains to acquire adaptive mutations. Further, we explored how *L. monocytogenes*
101 encoding a full-length InlA or an *inlA* gene with a PMSC were able to adapt and
102 subsequently resume growth at 4°C following downshift from 37°C.

MATERIALS AND METHODS

Bacterial isolates. *Listeria monocytogenes* used in this study were recovered from food processing environment (FPE) swabs (n=29), raw unprocessed food (RUF; n=6), and ready-to-eat (RTE) foods (n=19) that were collected from three dairy (DPF), five fish (FPF), and five meat (MPF) processing facilities across BC as part of a previous study (27). Using origin of isolation, serotyping, and pulsed-field gel electrophoresis (PFGE) data, we selected 54 different isolates for inclusion in this study. Isolate origins and serotyping data are described in Table 1. Isolates were serotyped by slide agglutination and antisera prepared according to Seeliger and Höhne (43) at the Canadian National Microbiology Laboratory, and PFGE was performed by the BCCDC Public Health Microbiology and Reference Laboratory. Bacterial cultures were maintained in peptone with 20% glycerol at -70°C. Prior to experiments, isolates were grown overnight on tryptic soy agar (TSA; Difco, Becton Dickinson Diagnostics, Mississauga, ON, Canada) at 35°C.

Internalin A DNA sequencing. Conventional polymerase chain reactions (PCR) were used to amplify the 2.4 kb *inlA* gene as described by Kovacevic et al. (28). Briefly, 5 U of AmpliTaq Gold 360 DNA polymerase (Invitrogen, Burlington, ON) was used with one set of custom primers (*inlA*-JK-F 5'-TAC AAC GAA ACC TGA TAT TG-3' and *inlA*-JK-R 5'-GCT AGA TAT AGT CCG AAA AC-3'), each at 0.5 mM, 200 mM dNTPs (Invitrogen), and 50-100 ng DNA template (50-100 ng) obtained using a DNeasy Blood and Tissue kit (Qiagen, Toronto, ON). Thermocycling was performed as follows: initial denaturation at 94°C for 2 min; 20 cycles of 94°C for 1 min, 60-50°C for 1 min with touchdown decrease of 0.5°C per cycle, and 72°C for 2.5 min; 20 cycles of 94°C for 1

126 min, 50°C for 1 min, and 72°C for 2.5 min; and a final extension step at 72°C for 7 min
127 (47). PCR product was purified using a QIAquick PCR Purification kit (Qiagen) and
128 sequenced at Canada's Michael Smith Genome Science Centre using the *inlA*-JK primer
129 set and previously published primers (47). Nucleotide sequences were assembled and
130 analyzed with Geneious 5.4 software (Biomatters Ltd., Aukland, New Zealand). The
131 presence of PMSCs was determined by comparing *inlA* sequence data to *L.*
132 *monocytogenes* EGD-e (19).

133 **Invasion of Caco-2 cells.** The invasion efficiency of seven representative *L.*
134 *monocytogenes* isolates from different serotypes, food and environmental samples, and
135 *inlA* genotypes were assessed in 24-well tissue culture plates according to Gaillard et al.
136 (16) with minor modifications. Briefly, Caco-2 cells (~2 x10^5 cells per well; passages 5 to
137 20) were cultured in Dulbecco's modified Eagles minimum essential medium (DMEM;
138 HyClone®, Thermo Scientific, Toronto, ON), supplemented with 10% inactivated fetal
139 calf serum (GIBCO, Life Technologies, Burlington, ON), 1% non-essential amino acids
140 (GIBCO), and 1% GlutaMAX (GIBCO) for 2 days (5% CO_2, at 37°C) to reach
141 confluency. Bacterial cultures grown statically overnight in BHI at 30°C were pelleted by
142 centrifugation (5,939 x *g*; Eppendorf 5415 R), washed once, re-hydrated in 1x
143 Dulbecco's phosphate buffered saline (DPBS; HyClone®) with magnesium and calcium,
144 and adjusted to OD_{600nm} = 0.5 (Genesys 10UV, Thermo Spectronic, Rochester, NY). Prior
145 to infection, cultures were diluted in DMEM to approximately 4 x 10^7 colony forming
146 units (CFU) per ml, as assessed by plating on TSA. Bacterial suspensions (0.5 ml) were
147 added to Caco-2 cells and incubated at 37°C for 1 h to allow bacterial entry. Cells were
148 washed three times, overlaid with fresh DMEM containing gentamicin (10 mg/l), and

incubated at 37°C for 2 h. Following incubation, the cell monolayer was washed three times with DPBS and treated with 1% Triton X-100 for 10 min at 37°C. The number of viable bacteria released was quantified by plating onto TSA. *L. monocytogenes* EGD-SmR and BUG5 (Tn*1545*-induced *inlA* mutant from EGD-SmR) (15), and 08-5578 (18), kindly provided by Dr. Pascale Cossart (Institut Pasteur) and Dr. Matthew Gilmour (Public Health Agency of Canada), respectively, were used as controls. The gentamicin concentration used (10 mg/l) was confirmed to kill all extracellular bacteria by plating inoculum onto TSA. Invasion assays for each isolate were carried out in triplicate and repeated two times.

Mutation frequency. Since differences in the occurrence of PMSCs exist amongst different serotypes, we examined how frequently isolates (n=54) acquired mutations following exposure to rifampicin (RIF) using previously published methodology described for Enterobacteriaceae with some modification (1, 29). Briefly, isolates were grown overnight at 35°C in BHI broth and adjusted to an OD_{600nm} = 1.0. A 100 µl aliquot was spread plated onto BHI with 100 µg/ml RIF. Following incubation for 48 h at 35°C, the number of CFU was counted. The assay for each isolate was carried out in triplicate and repeated two times. The mean number of colonies for all strains was determined, and comparisons made between strains with and without PMSCs and across serotypes.

Cold growth evaluation. A subset of isolates (n=33) representing *L. monocytogenes* with full-length *inlA*, 3-codon deletion (a.a. 738-740), and each type of PMSC observed in our collection was assessed for cold growth adaptation as described by Arguedas-Villa et al. (2). In short, a single colony was inoculated into 10 ml brain

heart infusion broth (BHI; Oxoid, Ottawa, ON) and grown overnight at 37°C with shaking (220 rpm) (~10^9 CFU/ml). Fresh BHI (10 ml) was inoculated with approximately 10^3 CFU/ml and incubated at 4°C until bacteria reached stationary phase. Growth was monitored by plating 10-fold serial dilutions prepared in peptone buffered saline onto plate count agar (Oxoid), incubating at 37°C for 24 h, and CFUs were counted. The lag phase duration (LPD) and exponential growth rate (EGR) of each strain were calculated from log converted growth (CFU/ml) data using Dmfit version 2.0 and Microfit version 1.0 programs, based on the models of Baranyi and Roberts (3).

Statistical analysis. Data analysis was performed using GraphPad Prism 6.0 software. The statistical significance of differences in *inlA* genotypes based on isolate source (FPE, RUF and RTE foods) was assessed using chi-square and Fisher's exact test (RUF and RTE foods). The Student's *t*-test was used to compare invasive *inlA* genotypes to control strains (08-5578, EGD-SmR or BUG5), and to examine whether differences exist in LPD and EGR between food and environmental strains. Mutability, as indicated by the number of RIF^R colonies, among serotypes (1/2a, 1/2c, 3a and 4b) was compared using Kruskal-Wallis test for nonparametric data, followed by Dunn's multiple comparisons test while differences between *inlA* genotypes (no PMSCs *vs.* PMSCs) were assessed by the Mann-Whitney test. A Fisher's exact test was performed to assess whether differences exist between cold adapting groups (fast, intermediate) and *inlA* genotypes (no PMSC *vs.* PMSCs). For all analyses, differences were considered significant if p was < 0.05.

RESULTS

195 ***inlA* genotypes and mutability amongst *L. monocytogenes*.** DNA sequencing of
196 *inlA* in 54 *L. monocytogenes* strains originating from food and FPE samples recovered
197 from dairy, fish, and meat processing facilities revealed PMSCs in 35% of isolates.
198 Isolates possessing truncated InlA due to PMSC mutations are hereafter referred to as
199 PMSC isolates. Type 3 mutations (amino acid [a.a.] position 700) were the most common
200 PMSC mutation in this collection (53%), followed by type 4 (32%) (a.a. position 9), type
201 11 (10%) (a.a. position 685), and only a single isolate (5%) possessed a type 1 mutation
202 (a.a. position 606) (Table 2) (47). Overall, 41% percent of isolates encoded a full-length
203 *inlA*, while 24% had a 3-codon deletion in a.a. positions 738 to 740 (aspartic acid,
204 threonine and serine), hereafter referred to as 3-codon deletion.

205 Since PMSCs have been frequently reported in serotype 1/2a strains, we
206 compared the mutability of 1/2a isolates to other serotypes, including 4b. Point mutations
207 occurring in the RIF resistance-determining region of *rpoB* may afford resistance to RIF
208 (RIF^R) (50). Following plating in the presence of 100 µg/ml RIF, the number of RIF^R
209 colonies ranged from 1.3 ± 0.7 to 52.8 ± 7.9 per plate. Irrespective of serotype,
210 significantly more RIF^R colonies were observed in strains not possessing PMSCs in
211 comparison to those with PMSC mutations (p=0.001) (Fig. 1A). Correspondingly,
212 significantly more RIF^R colonies were observed for 4b serotype strains compared to 1/2a,
213 1/2c, and 3a strains (p=0.0002) (Fig. 1B).

214 **Distribution of *inlA* genotypes across different food production facilities.** No
215 PMSCs were seen in *inlA* of *L. monocytogenes* isolates recovered from DPF, while 33%
216 and 60% of isolates from FPF and MPF, respectively, had PMSCs. Of five FPFs
217 examined, three facilities had isolates lacking PMSCs, while two facilities had PMSCs in

all (10/10) or 75% of recovered isolates (Table 3). More MPFs (n=3) had *L. monocytogenes* isolates possessing *inlA* PMSCs than isolates without mutations. Two MPFs had no PMSC mutations in their isolates, one had PMSCs in all isolates (n=3), and two facilities had 50% and 67% of isolates with mutations (Table 3). Type 3 mutations were seen amongst isolates from FPF and MPF, while type 4 was only seen in isolates from FPF, and type 1 and 11 only in *L. monocytogenes* from MPF. Type 3 mutations were the most common among isolates possessing PMSCs from FPF, followed by type 4 mutations. Similarly, type 3 mutations were most commonly seen in isolates from MFP, followed by type 11 and type 1 mutations (Table S1).

Isolates possessing a 3-codon deletion were seen in two FPFs (F20, F31), but not in DPFs or MPFs (Table 2). In one of the facilities (F20), all but one isolate (88%) had this deletion, while 60% of samples had the same codons missing in the other facility (F31). PFGE typing showed these isolates were not clonal (data not shown). None of the isolates from facilities F20 and F31 possessed PMSCs.

***inlA* mutations within different serotypes.** PMSC mutations were seen in four of the five serotypes examined, including all 1/2c (n=8) and 3a (n=4) isolates, followed by 1/2a (30%; n=6), and the only 1/2b (n=1) isolate examined. Serotypes 1/2a and 3a carried only type 3 mutations while serotype 1/2b had only a type 1 mutation. The only serotype with more than one type of mutation (i.e. type 4 and 11) was 1/2c.

A 3-codon deletion was observed in 13 strains deriving from FPFs. With the exception of one 1/2a isolate, this deletion was observed exclusively in 4b serotype strains. Overall, 57% of 4b serotype isolates possessed this 3-codon deletion, though no PMSC mutations were seen in serotype 4b isolates.

241 **Occurrence of *inlA* PMSCs in isolates recovered from different sources.**
242 Isolates with *inlA* PMSC mutations were seen more commonly in FPE samples than RUF
243 and RTE foods (p=0.01). Further, more isolates from RUF (67%) carried PMSCs
244 compared to those isolated from RTE (16%) foods (p=0.02). Nine of the 13 3-codon
245 deletion mutants were recovered from RTE food (69%), with the remaining isolates being
246 environmental. Isolates encoding a full-length InlA were seen predominantly in FPE
247 samples (55%), followed by RTE (36%) and RUF (9%) samples.

248 **Invasion of Caco-2 cells by *L. monocytogenes* possessing truncated InlA or a**
249 **3-codon deletion.** PMSC-encoding isolates exhibited reduced Caco-2 cell invasion
250 phenotypes (Fig. 2). A 4b isolate (FF46-3) possessing wild type *inlA* was 2.2 times more
251 invasive (p<0.0001) than a clinical isolate that claimed 23 lives during a 2008 deli meat
252 outbreak in Canada, and 10.8 times more invasive (p<0.0001) than the laboratory control
253 EGD-SmR strain (Fig. 2). This phenomenon was observed for another 4b isolate (FF19-
254 1) and a 1/2a strain (FE13-1) possessing the 3-codon deletion, both of which were 4.7
255 and 7.1 times more invasive (p<0.0001), respectively, compared to the control EDG-SmR
256 strain. When compared to the Canadian deli meat outbreak strain, FF19-1 and FE13-1
257 were as invasive or 1.4 times more invasive (p=0.006), respectively (Fig 2).

258 **Cold growth adaptation and growth of strains from different serogroups and**
259 **sources.** Three cold growth categories were observed among 33 isolates assessed for
260 their ability to adapt to 4°C following downshift from 37°C. The first category included
261 fast adapting strains (n=15) possessing a LPD less than 70 h. The second group was
262 comprised of intermediate cold growth adaptors with LPD ranging between 70 to 200 h,
263 and included the majority of strains (n=13). Finally, five strains adapted slowly to 4°C,

264 possessing a LPD > 200 h. Fast adapting strains included mainly RTE food-derived
265 isolates, and to a lesser degree, FPE and RUF isolates, whilst intermediate cold growth
266 adaptors were recovered predominantly from FPE, but also included isolates from RTE
267 and RUF foods (p>0.05) (Fig. 3). Slow growing strains were seen only in FPE and RTE
268 samples (Fig. 3). No significant differences were observed in LPD or EGR between food
269 and environmental isolates (Fig. 4).

270 The majority of fast adapting strains were serotype 4b (53%), followed by 1/2a
271 (40%) and 1/2c (7%) serotypes. Intermediate cold-adaptors were represented
272 predominantly by 1/2a strains (46%), followed by 1/2c, 4b, 3a and 1/2b serotypes,
273 respectively. Of the five slow adapting strains, three were 1/2a and two 4b serotypes.

274 **Cold growth adaptation of different *L. monocytogenes inlA* genotypes.**
275 Significant differences in the ability to adapt and grow at 4°C between isolates with and
276 without *inlA* PMSCs were observed (fast *vs.* intermediate, p=0.04). Overall, intermediate
277 cold adapting strains more frequently possessed *inlA* PMSCs (70%) compared to fast
278 (20%) and slow (10%) cold adaptors (Fig. 5A). In contrast, with the exception of two
279 isolates (serotype 1/2c and 1/2a), fast adapting strains lacked PMSCs (Fig. 5B). Notably,
280 isolates possessing a wild-type *inlA* (i.e. full length InlA) or the 3-codon deletion
281 comprised 57% of fast-adapting strains, followed by 26% intermediate, and 17% of slow
282 growing strains (Fig. 5A).

283 **DISCUSSION**

284 Our data demonstrate variability in *inlA* genotypes amongst *L. monocytogenes*
285 strains recovered from foods and food processing environments in BC, Canada that were
286 often unique within food processing facilities. Overall, 35% of strains possessed
287 mutations in *inlA* due to PMSCs, which is lower than the rate reported for food-chain
288 isolates in the United States (US) (45%) (8, 48), but similar to levels reported in France
289 (23). In addition to previously described *inlA* mutations, including type 1, 3, 4 and 11, we
290 observed *inlA* genotypes with a consecutive 3-codon deletion in the amino acid positions
291 738 to 740, a phenomenon to date reported only in a single isolate from a meat facility in
292 Portugal (14). It has been suggested that certain PMSC mutations accumulate at the
293 population level with notable differences in PMSCs occurring in North America
294 compared to European countries (41, 47, 48). Interestingly, we observed isolates with a
295 type 11 (a.a. 685) PMSC mutation, which to date have not been reported outside of
296 France (8, 13, 47, 48).

297 It is well established that frameshift and transition/transversion mutations in *inlA*
298 can lead to PMSCs, resulting in a truncated or non-secreted InlA. Strains possessing these
299 genotypes are associated with attenuated virulence (5, 25, 33) and are predominantly seen
300 in *L. monocytogenes* adapted to environmental and food processing niches (i.e. 1/2a
301 serotype strains), and to a lesser degree in clinical strains overrepresented by 4b serotypes
302 (23, 48). In-line with this, strains examined in our study possessing PMSC mutations
303 were 1/2a, 1/2c and 3a serotypes. In general, 4b strains are typically more conserved in
304 their genetic content, exhibiting lower recombination rates, and are less likely to possess
305 plasmids and extra-chromosomal elements (35, 36, 39). Interestingly, when we compared

306 different serotypes in their ability to acquire point mutations leading to RIF^R, 4b strains
307 were significantly (p=0.0002) more likely to gain mutations conferring resistance than all
308 other serotypes. Considering 1/2a serotype strains are known to possess mutations in
309 several virulence loci, including *actA*, *inlA*, and *prfA* (reviewed by Orsi et al. (35)), we
310 hypothesized 1/2a serotype strains would have higher mutability in this assay. In fact, the
311 opposite was observed, though reasons for this are not clear. This is particularly
312 interesting since positive selection, resulting from the acquisition of advantageous
313 mutations, has been reported to contribute to the evolution of numerous genes in 1/2a
314 strains but less often reported for 4b serotypes (11, 37). Although this assay has not
315 previously been used to examine mutability in *L. monocytogenes*, it has been used to
316 examine mutation rates in Enterobacteriaceae (1, 29) in which comparison to reference
317 strains was made to identify hyper-mutability. In our study, we examined the relative
318 ability of strains to acquire mutations leading to RIF^R. Although further work is needed to
319 explore this phenomenon, our results suggest serotype 4b strains may acquire mutations
320 more readily than 1/2a strains, demonstrating that there must exist selection pressure for
321 the maintenance of internalin A genes encoding a full-length InlA for serotype 4b.

322 Within our collection, we observed highly invasive isolates possessing a 3-codon
323 deletion (a.a. 738-740), and is contrary to a previous report (14). These isolates exhibited
324 invasion efficiencies equivalent to or surpassing that of the deli meat outbreak strain (08-
325 5578) that contributed to the deaths of 23 individuals in 2008 (49) and the EGD-SmR
326 strain (Fig. 2). In recent years, strains possessing truncated InlA proteins have been
327 identified as strains with lower invasiveness and, accordingly, suggested to present
328 reduced public risk (33, 34). Although this seems prudent for most *inlA* genotypes in

329 which PMSCs lead to truncated proteins, this does not apply to the 3-codon deletion we
330 observed. In contrast, although the InlA protein is truncated by three amino acids, isolates
331 possessing it remain equally or more invasive than control strains, indicating they are of
332 considerable risk and public health concern. Considering 12 of 13 3-codon deletions were
333 serotype 4b, we plan on comparing the internalin gene complement of the single 1/2a
334 isolate with this deletion to the 4b strains harboring the same *inlA* genotype. It has been
335 shown experimentally that the deletion of a.a. 714 to 766 corresponding to the pre-anchor
336 region did not reduce the invasiveness when the modified *inlA* gene had been transferred
337 to *L. innocua* (30). However, the impact of a consecutive deletion of aspartic acid,
338 threonine and serine in positions 738 to 740 in naturally occurring *L. monocytogenes*
339 strains has not been described. It is possible that this deletion may affect protein folding
340 in a manner enhancing bacterial interaction with its human cell surface receptor E-
341 cadherin. However, it is also possible that other virulence-related factors are contributing
342 to invasion. In particular, a host of other internalin genes (*inlB*, *inlC2*, *inlD*, *inlE*, *inlF*,
343 *inlG*, *inlH*) have been implicated in invasive behavior (35, 38, 45). Of these, *inlC2*, *inlD*,
344 *inlE*, and *inlJ* are common to lineage I and II strains, while *inlF*, *inlG*, and *inlH* have only
345 been observed in lineage II (35, 45).

346 In general, it has been proposed that over-representation of serotype 1/2a (lineage
347 II) strains in isolates originating from food and FPEs derives from enhanced capacity to
348 survive food chain conditions, though data substantiating this assertion are often
349 conflicting and limited (35). It has been reported, however, that 4b strains incubated at
350 4°C for four weeks and subsequently up-shifted to 37°C possessed shorter LPD than 1/2a
351 isolates (6). This implies 4b strains present in foods may quickly adapt to host

352 temperature, and correspondingly may be more likely to cause disease. In this study, we
353 assessed the ability of various serotypes to adapt and subsequently grow at 4°C following
354 a downshift from 37°C. In the 33 strains examined, we observed three cold-adapting
355 groups similar to observations previously made amongst *L. monocytogenes* derived from
356 different origins (2). When sample origin was examined, the majority of fast-adapting
357 strains were recovered from RTE foods, though differences were not significant. It is
358 tempting to speculate that wild type *inlA* genotypes (i.e. full-length) may be an indicator
359 of food chain strain fitness. Support for this stems from observations centering on the
360 absence/presence of *inlA* PMSCs in respective cold-growth groups. Significantly more
361 intermediate cold adaptor isolates possessed PMSCs (70%) in comparison to fast-
362 adapting isolates (p=0.04), with only two fast-adaptor isolates shown to encode a PMSC.
363 This observation lends support to the use of *inlA* as a suitable biomarker to identify high
364 risk strains, though in this light it may be used as an indicator of increased ability to adapt
365 and grow at refrigeration temperatures. Considering cold temperatures are used in RTE
366 food processing facilities and relied on to ensure product quality throughout the food
367 supply chain, these strains may possess enhanced ability to persist in FPE. Further, if
368 present in food, strains having an *inlA* gene producing a full-length InlA may have
369 increased ability to grow to unacceptable and potentially dangerous levels during cold
370 storage, particularly if abusive conditions are encountered.

371 **Conclusions.** In summary, we observed *inlA* mutations in four *L. monocytogenes*
372 serotypes recovered from the BC food continuum. Notably, when the mutability of
373 isolates was examined, serotype 4b isolates were shown to acquire mutations more
374 frequently than all other serotypes. Also, isolates with a 3-codon *inlA* deletion possessed

highly invasive phenotypes, suggesting this *inlA* genotype may be of public health concern. When we examined the ability of *L. monocytogenes* isolates to adapt to cold temperature, we demonstrated that isolates possessing rapid cold adaption were more likely to encode an *inlA* gene lacking PMSCs. Our findings substantiate in new ways the assertion that isolates lacking *inlA* PMSCs are a significant concern. In light of our findings which show these isolates were more commonly recovered from RTE food, possessed the capacity to more rapidly adapt and grow at refrigeration than isolates with PMSCs, and genetically possess a causally linked virulence determinant, they represent isolates of significant concern to food processors and public health officials.

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571 TABLE 1. Serotypes of *Listeria monocytogenes* recovered from food processing

572 environments, raw unprocessed, or ready-to-eat foods used in the study.

Source	*L. monocytogenes* Serotypes					
	Total	**1/2a**	**1/2b**	**1/2c**	**3a**	**4b**
Processing environment	29	11	1	5	4	8
Food[a]						
RUF	6	4	0	2	0	0
RTE	19	5	0	1	0	13
Total	**54**	**20**	**1**	**8**	**4**	**21**

[a]RUF, raw unprocessed food; RTE, ready-to-eat food products.

573 TABLE 2. Number of *L. monocytogenes* isolates recovered from food processing
574 environments (n=29), raw unprocessed (n=6) and ready-to-eat (n=19) foods with *inlA*
575 mutations resulting in premature stop codons (PMSC) or 3-codon deletions.

***inlA* Genotype**	**No. (%) environmental isolates**	**No. (%) food isolates**		**Serotype (No. of strains)**	**Facility ID**
		RUF	**RTE**		
Without PMSC[a]	16 (55)	2 (33)	17 (89)	1/2a (14), 4b (21)	D5, D7, D11, F20, F21, F28, M38, M44, M46, M49
With 3-codon deletion (a.a. 738 - 740)	4 (14)	0	9 (47)	1/2a (1), 4b (12)	F20, F31
With PMSC	13 (45)	4 (67)	2 (11)		
Type 1 (a.a. 606)	1 (3)	0	0	1/2b (1)	F49
Type 3 (a.a. 700)	7 (24)	2 (33)	1 (5)	1/2a (6), 3a (4)	F19, F21, F49, F50
Type 4 (a.a. 9)	5 (17)	0	1 (5)	1/2c (6)	F19
Type 11 (a.a. 685)	0	2 (33)	0	1/2c (2)	F38, F50

576 [a]Includes isolates with the 3-codon deletion (a.a. 738-740)

577

578 TABLE 3. Distribution of *L. monocytogenes* isolates (n=54) and the presence of *inlA*
579 premature stop codons across dairy (DPF), fish (FPF), and meat (MPF) processing
580 facilities (n=13).

ID/Type Facility Type		Total no. isolates	Isolate source[a]			*inlA* (positive/tested)	
			FPE	RUF	RTE	No PMSC[b]	With PMSC
D5	DPF	3	3	-	-	3/3	-
D7	DPF	1	1	-	-	1/1	-
D11	DPF	1	1	-	-	1/1	-
F19	FPF	10	9	-	1	-	10/10
F20	FPF	8	4	-	4	8/8	-
F21	FPF	4	2	-	2	1/4	3/4
F28	FPF	7	4	-	3	7/7	-
F31	FPF	10	1	-	10	10/10	-
M38	MPF	2	-	2	-	1/2	1/2
M44	MPF	1	-	1	-	1/1	-
M46	MPF	1	1	-	-	1/1	-
M49	MPF	3	3	-	-	1/3	2/3
M50	MPF	3	-	3	-	-	3/3

581 [a]FPE, food production environment; RUF, raw unprocessed food, RTE, ready-to-eat food;
582 [b] PMSC, premature stop codon.

FIG. 1. Mutability of different *L. monocytogenes inlA* genotypes (A) and serotypes (B) assessed by the number of rifampicin-resistant colonies after 48 h growth at 35°C in the presence of 100 µg/ml rifampicin. Mutability of each isolate was assayed in triplicate in each experiment, and two independent experiments were performed. Bars represent mean number of colonies, and error bars indicate standard error of the mean. Different letters above the bars represent significant differences ($p<0.05$) between geno- and serotype groups determined using the Mann-Whitney (A) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (B). Serotype 1/2b strains were excluded from statistical analysis as only one isolate was recovered.

FIG. 2. Invasion efficiency (% of bacteria recovered relative to initial inoculum) of *L. monocytogenes* isolates possessing *inlA* PMSC mutations (type 1, 3, 4 and 11) or a 3-codon deletion at amino acid position 738 to 740 (Δ738-740) in comparison to wild type clinical isolates (08-5578 and EGD-SmR) and a Tn*1545*-induced non-invasive *inlA* mutant of EGD-SmR (BUG5). Assays for each isolate were carried out in triplicate and repeated two times. Bars represent mean invasion efficiencies, and error bars indicate standard error of the mean. Different symbols above the bars indicate significantly higher invasion efficiency ($p<0.005$; *t*-test) when compared to controls 08-5578 (●), EGD-SmR (♦) or BUG5 (■).

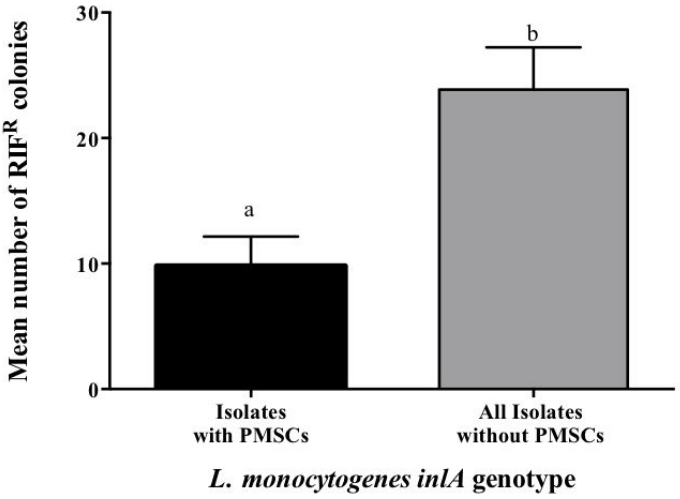
FIG. 3. Cold growth adaptation of *L. monocytogenes* isolates recovered from food processing environments (FPE), raw unprocessed (RUF), and ready-to-eat (RTE) foods, when grown at 4°C. Differences were not statistically significant ($p>0.05$, chi-square).

FIG. 4. Lag phase duration (A) and exponential growth rate (B) of 33 *L. monocytogenes* isolates recovered from food and food processing environments following a down-shift from 37 to 4°C in BHI. Each isolate was assayed in duplicate, and two independent growth assays were performed. The scatter plots are displaying mean values with standard deviation.

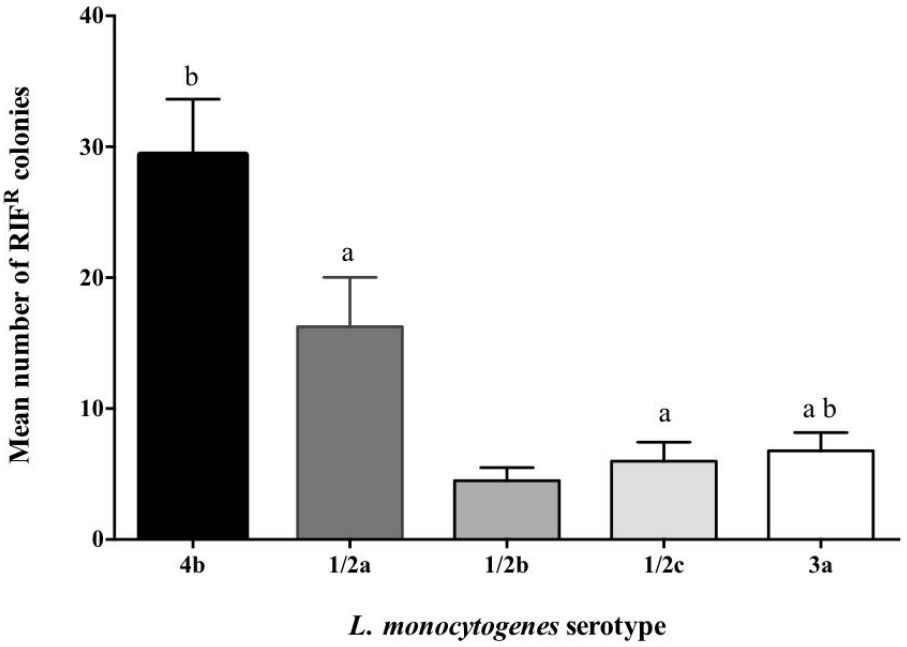
FIG. 5. Distribution of *inlA* genotypes (i.e. PMSC versus no PMSC) (A) and identification of cold adaptive groups (i.e. fast, intermediate, or slow) (B) observed at

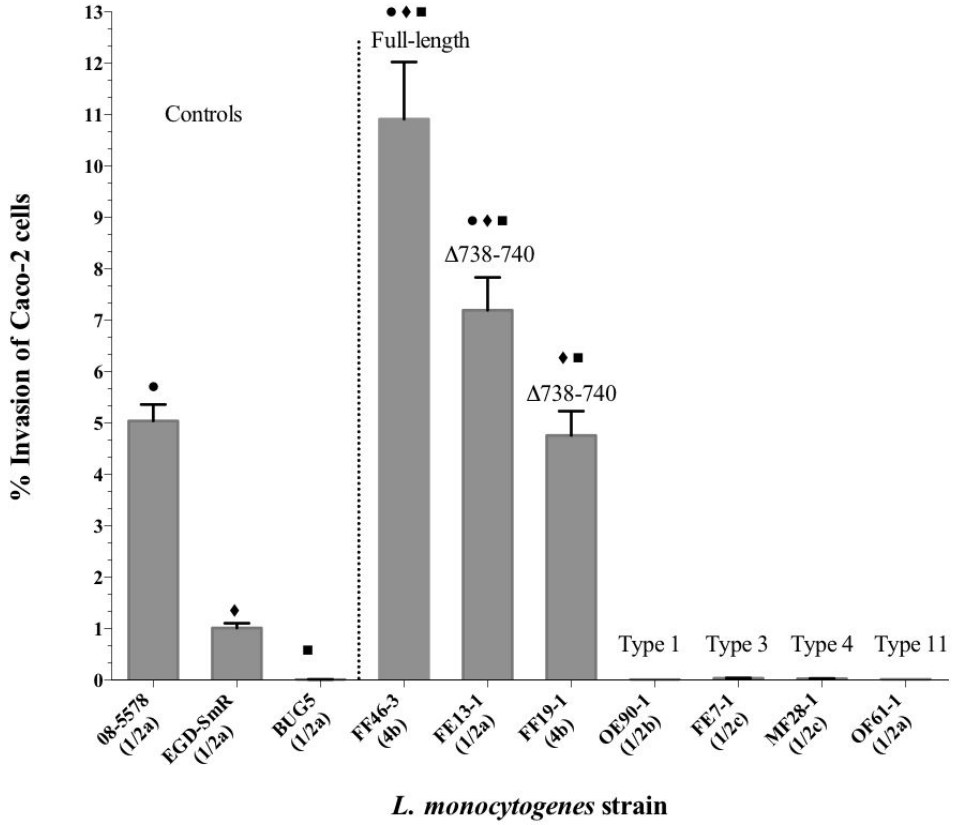
4°C following a downshift from 37°C in BHI. Differences in cold growth adaptation between fast and intermediate *L. monocytogenes inlA* genotypes were significant (Fisher's exact, p=0.04). The percentage of isolates within respective *inlA* genotypes (A) and cold adaptive groups (B) is indicated above bars.

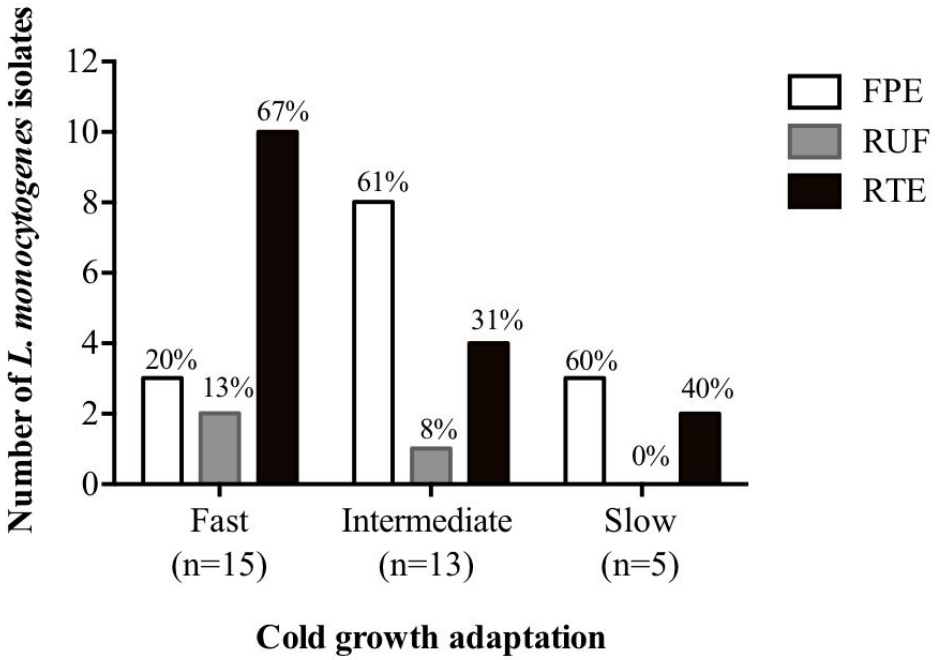
A.



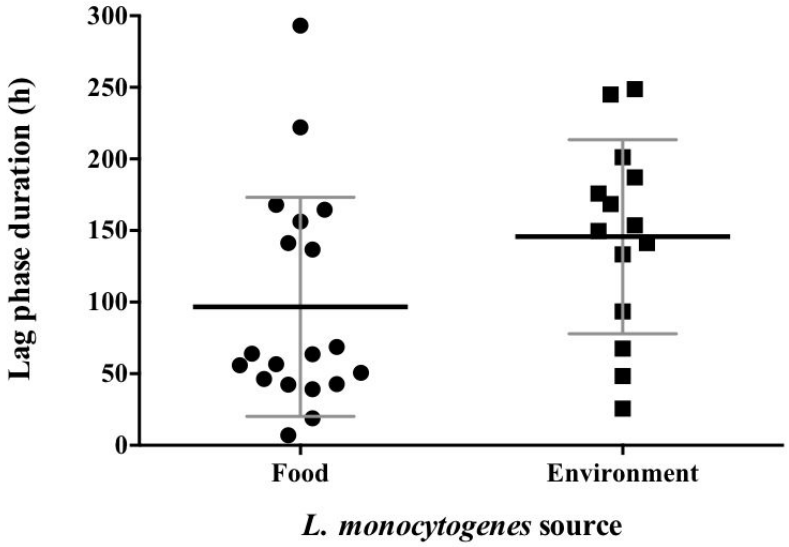
B.



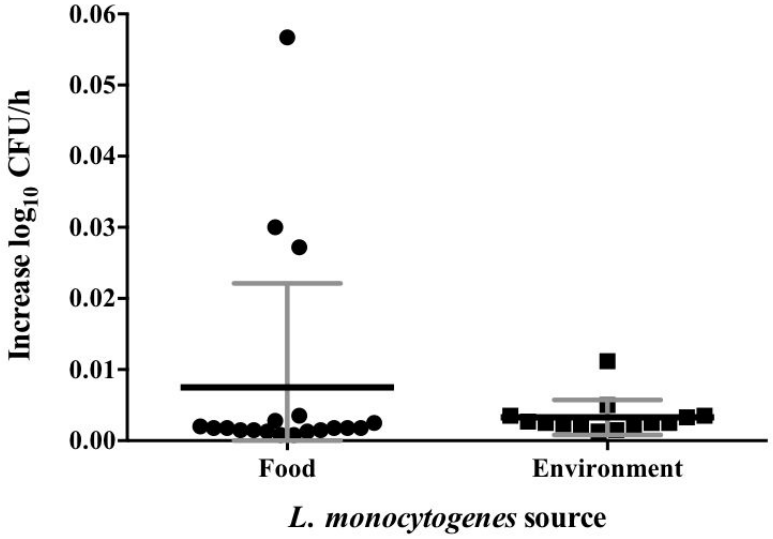




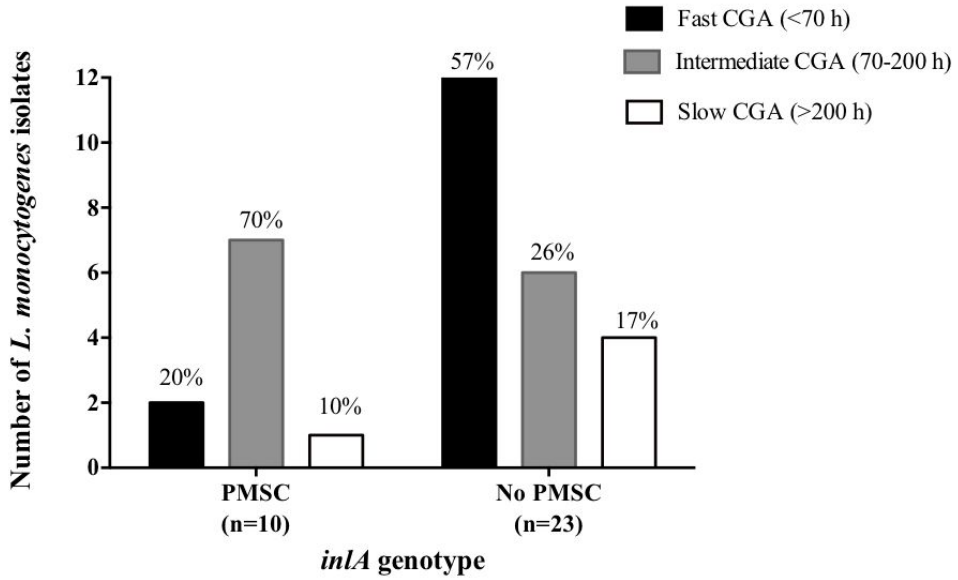
A. LPD



B. EGR



A.



B.